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(54) Title: METHOD FOR THE DETECTION OF VIABLE CRYPTOSPORIDIUM PARVUM OOCYSTS

(57) Abstract

Oligonucleotide molecules and methods are disclosed for the detection of viable oocysts or other cells of the protozoa species, *Cryptosporidium parvum*. Preferred oligonucleotide molecules are selected from the group comprising oligonucleotides having one or more of the following sequences; (a) ACA ATT AAT, (b) CTT TTT GGT, (c) AAT TTA TAT AAA ATA TTT TGA TGA A, (d) TTT TTT TTT TTA GTA T, e) TAT ATT TTT TAT CTG, (f) CTT TAC TTA CAT GGA TAA CCG, or comprising a part of the sequences (a) to (f) above so as to allow specific hybridisation to unique 18S rRNA sequences of *C.parvum*.

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# METHOD FOR THE DETECTION OF VIABLE CRYPTOSPORIDIUM PARVUM OOCYSTS

## Field of the Invention

The present invention relates to a method for the detection of viable oocysts or other cells of the protozoa species, *Cryptosporodium parvum*. More particularly the invention relates to a method for such detection utilising one or more of a number of probes capable of hybridising to specific *C.parvum* 18S rRNA sequences.

## Background Art

The detection of *Cryptosporidium* oocysts in water presently relies on the concentration of particulate matter from large volumes of water prior to staining with fluorescently labelled monoclonal antibodies. Until recently, detection and identification of fluorescently labelled oocysts required examination of the sample using epifluorescence microscopy. The tedious and labour intensive nature of this detection method, in particular the amount of fluorescent microscopy required, limited the monitoring work which could be performed. The development of flow cytometric assisted detection methods has alleviated some of these problems and enabled the routine monitoring of water for the presence of *Cryptosporidium* oocysts (Vesey *et al.*, 1994A). However, a major limitation of all these methodologies is the lack of oocyst viability measurements. Methods to determine viability such as animal infectivity and excystation, are impractical because of the low number of oocysts normally present in water samples and the tedious nature of such tests.

The presence of dead *Cryptosporidium* oocysts in drinking water is of little significance to public health, however if oocysts are viable the risk to public health is enormous. Moreover, of the seven species of *Cryptosporidium*; *C.parvum, C.muris, C.meleagridis,*

*C.serpentis, C.nasorum, C.wrairi and C.baileyi* only one, *C.parvum* is infectious to humans (Rose *et al.*, 1988), and yet all the currently available monoclonal antibodies are not species specific. There is, therefore, an urgent requirement to develop an effective method for determining the viability of *C.parvum* oocysts in water.

Fluorescence *in situ* hybridisation (FISH) is a relatively new method by which microorganisms can be specifically labelled. The technique is reliant upon the identification of a specific sequence of nucleic acid within the target organism. Probes targeting a specific nucleic acid sequence are then synthesised and labelled with a fluorochrome. The cell is then permeabilised and the complementary sequence allowed to hybridise with the target sequence resulting in specific labelling of the target cell.
The use of ribosomal RNA (rRNA) targeted oligonucleotide probes with FISH and flow cytometry has been reported by Amann *et al.* (1990B). Molecules of rRNA are ideal targets for fluorescently labelled nucleic probes for several reasons: (1) high sensitivity can be achieved since the target molecules are present in very high numbers; (2) a denaturation step is not required during the procedure as the target region is single stranded; and (3) rRNA has a short half life and will only be present in a high copy number in viable cells.

**Disclosure of the Invention**

In a first aspect, the present invention provides an oligonucleotide molecule for the detection of viable cells of *Cryptosporidium parvum* (*C.parvum*), the oligonucleotide molecule characterised in that it specifically hybridises to unique 18S rRNA sequences of *C.parvum*.

Preferably, the oligonucleotide molecule specifically hybridises to unique 18S rRNA sequences of *C.parvum* under medium to high stringency conditions (Sambrook *et al.*, 1989.), however, in most cases, conditions of high stringency will be required to ensure specific hybridisation to unique *C.parvum* 18S rRNA sequences.

In a preferred embodiment of the invention, the oligonucleotide molecule is selected from the group comprising oligonucleotides having one or more of the following nucleotide sequences:

(a) ACA ATT AAT
(b) CTT TTT GGT
(c) AAT TTA TAT AAA ATA TTT TGA TGA A
(d) TTT TTT TTT TTA GTA T
(e) TAT ATT TTT TAT CTG
(f) CTT TAC TTA CAT GGA TAA CCG

or comprising a part of the sequences (a) to (f) above so as to allow specific hybridisation to unique 18S rRNA sequences of *C.parvum*.

Preferably, the oligonucleotide molecules according to the invention are detectably labelled so that they may be utilised as probes in hybridisation assays. However, oligonucleotide molecules which are not labelled may be used in a polymerase chain reaction (PCR) to amplify a part of the rRNA of *C.parvum* to allow its detection.

In a second aspect, the present invention consists in a method for the detection of the presence of viable cells of *C.parvum* in a sample, comprising the steps of adding to the sample a probe consisting of a detectably labelled oligonucleotide molecule according to the first aspect of the invention; permeabilising or lysing the cells present in the sample to allow hybridisation of the probe to the 18S rRNA of any *C.parvum* cells present in the sample; and detecting the hybridisation of the probe to cells in the sample.

In a preferred embodiment, the second aspect of the invention is used in combination with fluorescence *in situ* hybridisation (FISH) in which the oligonucleotide probe is labelled with fluorochrome and the resulting fluorescent cell is detected by flow cytometry.

In a further preferred embodiment of the invention, several different oligonucleotide probes are used and are distinguished by the use of different labels. More preferably the oligonucleotide probes are labelled with different fluorochromes and detected by flow cytometry.

While it is preferred that the probes are fluorescently labelled it is to be understood that other known forms of labelling may be used within the broad scope of the present invention. Examples of other forms of labelling are radioactivity and chemiluminescence.

The oligonucleotide molecules and methods of the invention, may be used to detect the presence in a sample of any type of viable cell of *C.parvum*. Normally only oocysts will be found in environmental samples. Other cell types may, however, be found and detected in clinical samples.

**Brief Description of the Drawings**

Figure 1 shows the results of flow cytometric analysis of FISH stained oocysts using a eukaryotic specific probe (Euk probe) and a bacterial specific probe (Bac probe).

Figure 2 shows the comparison of oocyst viability as measured by excystation and viability determined by FISH on oocysts following storage at 22°C in the dark and sampled over a 74 day period.

Figure 3 shows the position of the specific *C.parvum* 18S rRNA probes in respect of the whole 18S rRNA sequence. The various sequences shown are:

Sequence 1: *C.parvum*, 18S complete, 1750 bp, L16997.

Sequence 2: *C.muris*, 18S complete, 1743 bp, L19069.

Sequence 3: *C.baileyi*, 18S complete, 1733 bp, L19068.

Sequence 4: *C.parvum*, Maquarie Uni, 18S, primer 180R.

## Best Mode of Carrying out the Invention

## MATERIALS AND METHODS

### Cryptosporidia

**Cryptosporidium parvum**. *Cryptosporidium parvum* oocysts cultured in lambs and purified by density gradient centrifugation were purchased from the Moredun Animal Research Institute, Edinburgh, UK.

### Eukaryotic Specific and Eubacterial Specific Probes

**Oligodeoxynucleotide probes.** A probe (Euk) complementary to a 18S rRNA region conserved for Eucarya (5'-ACCAGACTTGCCCTCC-3') (Amann *et al.*, 1990) was used to stain *Cryptosporiduium* oocysts. A second probe (Bac) complementary to a 16S rRNA region conserved for all bacteria (5'-GCTGCCTCCCGTAGGAGT-3') (Amann *et al.*, 1990) was used as a negative control for non-specific binding in all experiments. The probes were synthesised and labelled with fluorochromes as described previously (Wallner *et al.*, 1993).

### Design of a Specific FISH-Probe for C.parvum

**Extraction of genomic DNA**: Oocysts were pelleted by centrifugation, resuspended in TE buffer, and repeatedly frozen in a mixture of dry ice and ethanol and thawed by boiling for 2 mins. After incubation with SDS and proteinase K (1% w/v 100 μg/ml respectively)for 1 h at 37°C the lysate was extracted with phenol, phenol-chloroform and chloroform-isoamylalcohol. Nucleic acid was precipitated with 1 volume 4M ammonium acetate pH 4.5 and 2 volumes isopropanol, washed with 70% ethanol, dried, and finally dissolved in distilled water.

**Sequence analysis and probe/primer design.** Complete 18S rRNA sequences comprising the species *C.parvum*, *C.muris* and *C.baileyi* were obtained through the EMBL and GenBank data bases. The sequences were manually aligned. One particular region appeared to have the potential to

discriminate *C.parvum* from the other species included in the analysis. To examine the validity of the published 18S rRNA sequence of *C.parvum* a short stretch of *C.parvum* rDNA including one of the putative probe regions (Table 1, Number 6) was sequenced. Two primers targeting the conserved 5'- and 3'-end of the 18S rDNA were designed (5'AAC CTG GTT GAT CCT GCC FORWARD and 3'GGT TCA CCT ACG GAA ACC REVERSE) and employed to amplify the gene via PCR as described previously for 16S rDNA (Dorsch and Stackebrandt, 1992). The probe region was then sequenced using a reverse primer (CCT TCC ATA AAG TCG AGT) complementary to a sequence approximately 50 nucleotides downstream. The sequencing protocol was as described (Dorsch and Stackebrandt, 1992). Our results clarified the ambiguity in the literature concerning the published *C.parvum* 18S rRNA sequence (see Fig. 3). The accessibility of the target region within native ribosomes for fluorescence *in situ* hybridisation was estimated using a secondary structure model for the 18S rRNA of angiosperms (Schmidt-Puchta *et al.*, 1989), and six sequences were identified as being specific to *C.parvum* (Table 1).

**TABLE 1.** Potential targets on the 18S ribosome for *Cryptosporidium parvum* specific probes. The position of the target sites with respect to the whole 18S rRNA sequence are shown in Figure 3.

| Number | Sequence |
|---|---|
| a | ACA ATT AAT |
| b | CTT TTT GGT |
| c | AAT TTA TAT AAA ATA TTT TGA TGA A |
| d | TTT TTT TTT TTA GTA T |
| e | TAT ATT TTT TAT CTG |
| f (CRY1) | CTT TAC TTA CAT GGA TAA CCG |

A fluorescently labelled probe was designed for sequence number 6 (CGG TTA TCC ATG TAA GTA AAG).

**Oligonucleotide synthesis.** Oligonucleotides of the *C.parvum* specific probe number 6 (CRY1) were synthesised and labelled with FITC by Biotech Int. (Perth).

**Sample Preparation**

**Fixation of oocysts.** Fixation of oocysts was performed using a modified method of that described previously by Wallner *et al.* (1993) for the fixation of yeasts and bacteria. One volume of oocyst suspension containing approximately $10^7$ oocysts, was mixed with three volumes of fresh cold 4% w/v paraformaldehyde in phosphate buffered saline (PBS), pH 7.2, and kept at 4°C for 1 h. The oocysts were washed three times by centrifugation (13,000 g, 30 s) and then resuspended in PBS. The sample was then mixed with an equal volume of cold (-20°C) absolute ethanol and hybridised within 1 h or stored at 4°C.

**Hybridisation.** Fixed oocysts were hybridised with the probe by mixing 10 µl of oocyst suspension with 100 µl of hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.5% w/v sodium dodecylsulfate) prewarmed to 48°C and then 10 µl of probe added (25 ng/µl in distilled water). The sample was mixed and incubated at 48°C for 1 h. The sample was then washed by centrifugation (13,000 g, 30 s) and resuspended in hybridisation buffer, without sodium dodecylsulfate, prewarmed to 48°C. Samples were then analysed immediately using flow cytometry or epifluorescence microscopy.

**Sample analysis.** Flow cytometry was performed using a Coulter Elite flow cytometer or a Coulter XL flow cytometer as described previously (Vesey *et al.*, 1993; Vesey *et al.*, 1994A; Vesey *et al.*, 1994B).

Epifluorescence microscopy was performed using a Nikon Optiphot-2 microscope fitted with differential interference contrast (DIC) optics and

excitation and emission filters suitable for the examination FITC. Oocysts were detected using DIC and then examined for fluorescence. A minimum of 100 oocysts were examined in each sample.

**Excystation.** *In vitro* excystation was performed as described by Campbell *et al.* (1992). To a 100 µl volume of oocyst suspension (approximately $10^4$ oocysts), 10 µl of 1 (w/v) sodium deoxycholate in Hanks minimal essential medium and 10 µl of 2.2% sodium hydrogen carbonate in Hanks balanced salt solution are added. After incubation, 37°C for 4 h, samples were examined microscopically using DIC optics. The proportion of empty oocysts, partially excysted oocysts and non-excysted oocysts were determined. At least 100 oocysts were counted in each sample. The percent excystation was calculated as follows:

$$\frac{\text{(number of empty oocysts + number of partially excysted oocysts)}}{\text{total number of oocysts counted}} \times 100$$

where the number of empty oocysts equalled the number pre-excystation subtracted from the number post excystation.

**Stored samples.** To determine if samples could be fixed and then stored before analysis, storage experiments were performed. Aliquots (100 µl) of fixed oocyst suspensions were stored at 4°C for 1 month. Samples were removed at time intervals, stained with FISH using the eukaryotic specific probe and analysed using flow cytometry. All experiments were performed in triplicate.

**Aging of oocysts.** Aliquots (10 µl) of oocyst suspensions containing $10^8$ oocysts were diluted in 10 ml of PBS and stored at 22°C in the dark. Samples (0.5 ml) were taken at time intervals and the viability of oocysts assessed using both FISH with the eukaryotic specific probe and excystation. All experiments were performed in triplicate.

RESULTS

**Staining of oocysts with FISH.** Microscopic examination of oocysts, which had been stained using fluorescence *in situ* hybridisation with the Euk rRNA probe, revealed brightly fluorescent oocysts together with oocysts which showed no fluorescence. Fluorescent staining was located within the sporozoites. Examination of the fluorescent oocysts using DIC optics revealed intact oocysts with a small gap between the oocyst wall and the internal structures. In comparison, non-fluorescent oocysts frequently appeared to have a ruptured oocyst wall and a large gap between the oocyst wall and the internal structures. Empty oocysts did not fluoresce.

Flow cytometric analysis of oocysts stained by FISH with the Euk rRNA probe resulted in two distinct populations, a brightly fluorescent population and a non-fluorescent population indicating viable and non-viable populations respectively. This is illustrated in the scatter plot (Figure 1) in which the Y axis represents fluorescence and the X-axis side scatter. Analysis by epifluorescence microscopy and flow cytometry of samples which had been stained with the Bac probe resulted in no fluorescence in any oocysts above that of the autofluorescence of unstained occysts (Figure 1).

**Stored samples.** Samples of fixed oocysts which had been stored at 4°C for up to 4 weeks and then stained with FISH using the Euk probe showed no reduction in the number of oocysts which fluoresced and no reduction in the brightness of fluorescence (Table 2).

TABLE 2. A comparison of the fluorescence intensity of freshly fixed oocysts and oocysts fixed and stored at 4°C for up to 3 weeks, before staining with FISH and analysed using flow cytometry.

| Day | Mean Fluorescence |
|---|---|
| 0 | 17.1 |
| 7 | 17.3 |
| 14 | 16.6 |
| 21 | 17.8 |

**Comparison of viability determined by FISH and in vitro excystation.** A comparison of oocyst viability, measured by excystation and staining by FISH with the Euk and CRY1 probes on batches of oocysts obtained from Moredun Animal Research Institute are presented in Table 3. Results were very similar for both methods of assessing oocyst viability indicating that the rRNA probes only stain viable oocysts.

**TABLE 3.** Comparison of oocyst percentage viability determined by excystation and viability determined by FISH on three different batches of oocysts.[1]

| | Batch 1 (Euk) | Batch 2 (Euk) | Batch 3 (Euk) | Batch 4 (CRY1) |
|---|---|---|---|---|
| **FISH** | 56% (1.2)[(2)] | 76% (3.7) | 95% (2.2) | 71% |
| **Excystation** | 54% (2.7) | 79% (2.8) | 92% (4.9) | 69% |

[1]100 oocysts were examined for each determination.

[2]Standard Deviation.

Comparison of FISH and excystation for determining oocyst viability on suspensions of oocysts stored at 22°C in the dark are presented in Figure 2. Results are very similar for both methods. A gradual decline in the viability of the oocysts from 90% to 40% over the 74 day period was observed with both methods. Correlation of the two sets of results was highly significant, with a calculated correlation coefficient (r) of 0.998.

The present inventors have developed a method using FISH and a rRNA directed probe to assess the viability of *Cryptosporidium sp.* oocysts. However, the invention is not restricted to oocysts. The unique 18S rRNA sequence of *C.parvum* identified and the method of the detection as stated, may also be used to detect other life stages of the *C.parvum* organism.

The preferred method for detecting the unique 18S rRNA sequences of *C.parvum* in an environmental sample is the use of labelled oligonucleotide probes.

Oocysts containing fluorescing sporozoites after hybridisation with the probes are viable and oocysts which do not fluoresce are dead. The reason that dead oocysts do not stain is because the rRNA which the probes bind to deteriorates rapidly and in dead oocysts is not present in sufficient copy numbers to be detected.

It is envisaged that the PCR may be used to detect viable *C.parvum* cells. In a PCR reaction, one of the specific oligonucleotide molecules, as disclosed, may be used in combination with a second oligonucleotide molecule (which may or may not be specific to *C.parvum*) to amplify 18S rRNA sequences from *C.parvum*. The amplified sequence will contain at least part of one of the unique sequences of *C.parvum*. The oligonucleotides in the PCR reaction may also be labelled. The employment of PCR to detect viable *C.parvum* would be useful in analysis of body fluids or excretions from animals, including humans.

*In vitro* excystation is currently considered the gold standard to which methods for determining oocyst viability are compared. Results from comparing measurement of oocyst viability using FISH and measuring viability using *in vitro* excystation produced very similar results with both methods for all samples of oocysts analysed. Correlation of the FISH assay with excystation was highly statistically significant, with a calculated correlation coefficient of 0.998. Furthermore, the FISH method was found to be easy to perform and the results easily interpreted. Oocysts were either

fluorescent, indicating a viable oocyst, or did not fluoresce at all, indicating a dead oocyst.

FISH techniques have the potential to alleviate the problems of assaying environmental water samples through their increased specificity and are ideal staining methods for analysis by flow cytometry.

Another significant advantage of the FISH method is that samples can be fixed and then stored at 4°C prior to analysis. By contrast, samples which are to be analysed using a known method must be analysed immediately as storage or fixation of the samples will result in a reduction in the viability of the oocysts as measured by this technique.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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**Claims**:-

1. An oligonucleotide molecule for the detection of viable cells of *Cryptosporodium parvum (C.parvum)*, the oligonucleotide molecule characterised in that it specifically hybridises to unique 18S rRNA sequences of *C.parvum*.

2. An oligonucleotide molecule according to claim 1, wherein said oligonucleotide molecule specifically hybridises to unique 18S rRNA sequences of *C.parvum* under medium to high stringency conditions.

3. An oligonucleotide molecule according to claim 1 or 2, wherein said oligonucleotide molecule specifically hybridises to unique 18S rRNA sequences of *C.parvum* under high stringency conditions.

4. An oligonucleotide molecule according to any one of the preceding claims, the oligonucleotide molecule being selected from the group comprising oligonucleotides having one or more of the following nucleotides sequences:

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(a) ACA ATT AAT
(b) CTT TTT GGT
(c) AAT TTA TAT AAA ATA TTT TGA TGA A
(d) TTT TTT TTT TTA GTA T
(e) TAT ATT TTT TAT CTG
(f) CTT TAC TTA CAT GGA TAA CCG

or comprising a part of the sequences (a) to (f) above so as to allow specific hybridisation to unique 18S rRNA sequences of *C.parvum*.

5. An oligonucleotide molecule consisting of the sequence: ACA ATT AAT.

6. An oligonucleotide molecule consisting of the sequence: CTT TTT GGT.

7. An oligonucleotide molecule consisting of the sequence: AAT TTA TAT AAA ATA TTT TGA TGA A.

8. An oligonucleotide molecule consisting of the sequence: TTT TTT TTT TTA GTA T.

9. An oligonucleotide molecule consisting of the sequence: TAT ATT TTT TAT CTG.

10. An oligonucleotide molecule consisting of the sequence: CTT TAC TTA CAT GGA TAA CCG.

11. A method for the detection of the presence of viable cells of *Cryptosporodium parvum (C.parvum)* in a sample, comprising the steps of adding to the sample a probe consisting of a detectably labelled oligonucleotide molecule according to any one of the preceding claims; permeabilising or lysing the cells present in the sample to allow hybridisation of the probe to the 18S rRNA of any *C.parvum* cells present in the sample; and detecting the hybridisation of the probe to cells in the samples.

12. The method according to claim 6 used in combination with fluorescence *in situ* hybridisation (FISH), wherein the oligonucleotide probe

molecule is labelled with fluorochrome and detection of hybridisation is by flow cytometry.

13. A method according to claim 11 or 12, wherein several different oligonucleotide probe molecules are used and are distinguished by the use of different detectable labels.

14. The method according to claim 13, wherein the oligonucleotide probe molecules are labelled with different fluorochromes.

15. A method for the detection of the presence of viable cells of *Cryptosporidium parvum (C.parvum)* in a sample, comprising the steps of adding to the sample first and second primers, said first primer consisting of an oligonucleotide molecule according to any one of claims 1 to 10; permeabilising or lysing the cells present in the sample to allow hybridisation of the primers to the 18S rRNA of any *C.parvum* cells present in the sample; conducting a polymerase chain reaction (PCR) to amplify a part of the 18S rRNA of *C.parvum*; and detecting the amplified part of the rRNA in the sample.

16. A method according to claim 15, wherein said second primer is a second oligonucleotide molecule according to any one of claims 1 to 10.

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00274

**A. CLASSIFICATION OF SUBJECT MATTER**

Int Cl[6]: C12Q 1/68 C07H 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC : C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT : cryptosporid: or C( ) parvum
CHEMICAL ABSTRACTS : [cryptosporid: or C ( ) parvum] and [18S (RRNA) or 18S ( ) RIBOS: ( ) RNA]
CHEMICAL ABSTRACTS : nucleic acid sequence search

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 95/04749 A (ISIS PHARMACEUTICALS, INC) 16 February 1995<br>SEQ ID NO 69 page 45 | 1-5 |
| X | WO 95/02605 A (FLORIDA STATE UNIVERSITY) 26 January 1995<br>SEQ ID NO 13, Fig 2 | 1-4, 6 |
| X | WO 94/05333 A (ISIS PHARMACEUTICALS, INC) 17 March 1994<br>SEQ ID NO 65, page 63 | 1-5 |

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
8 July 1996

Date of mailing of the international search report
18 JUL 1996

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# INTERNATIONAL SEARCH REPORT

| I.. ..national Application No. |
|---|
| PCT/AU 96/00274 |

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 93/15221 A (HITACHI CHEMICAL CO. LTD et al.) 5 August 1993<br>SEQ ID NO 184, page 46 | 1-5 |
| X | EP 544212 A1 (NISSHIN FLOUR MILLING CO. LTD) 2 June 1993<br>pages 5 line 23 | 1-5 |
| X | WO 92/12992 A (JAMES N. GAMBLE INSTITUTE OF MEDICAL RESEARCH) 6 August 1992<br>claims 70-74, SEQ ID NO 15 page 162, SEQ ID NO 26 page 165 | 1-4, 6 |
| X | WO 92/02625 A (PUBLIC HEALTH LABORATORY SERVICE BOARD) 20 February 1992<br>claim 23 page 41 | 1-5 |
| X | WO 91/08312 A (GENE-TRAK SYSTEMS) 13 June 1991<br>probe no. 6-2, fig 3 | 1-5 |
| X | WO 91/06643 A (MAJESTY (HER) IN RIGHT OF CANADA as represented by THE NATIONAL RESEARCH COUNCIL OF CANADA) 16 May 1991<br>claims 7, 9, oligonucleotides (AseI) | 1-5 |
| X | EP 206769 A2 (FUJISAWA PHARMACEUTICAL CO. LTD) 30 December 1986<br>page 19 oligonucleotide 7 (G) | 1-5 |
| X | WO 86/05805 A (NORDISK GENTOFTE A/S) 9 October 1986<br>claim 4 page 11 | 1-5 |
| X<br>Y | Awad-El-Kariem F.M. et al., Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. Parasitology (1994), 109, 19-22<br>entire document<br>entire document | 1-3, 11-16<br>4-10 |
| P,X | Xigang Leng et al. Differentiation of *Cryptosporidium parvum, C.muris,* and *C.baileyi* by PCR-RFLP analysis of the 18S rRNA gene. Veterinary Parasitology 62 (1996) 1-7<br>entire document | 1-3, 11-16 |
| Y | Johnson A.M. et al., Phylogentic relationships of *Cryptosporidium* determined by ribosomal RNA sequence comparison, Int. J. Parasitol., 20(2), (1990) 141-147<br>entire document | 1-16 |
| X<br>Y | Cai J. et al., PCR cloning and nucleotide sequence determination of the 18S rRNA genes and internal transcribed spacer 1 of the protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium muris.* Biochimica et Biophysica Acta, 1131 (1992) 317-320<br>entire document<br>entire document | 1-3, 11-16<br>4-10 |
| Y | Kilani R.T et al., Geographical variation in 18S rRNA gene sequence of *Cryptosporidium parvum*, Int. J. Parasitol, 24(2), (1994), 303-306<br>entire document | 1-16 |

## INTERNATIONAL SEARCH REPORT

International Application No.
PCT/ AU 96/00274

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Vessey G et al. "Detection of Specific Microorganisms in environmental samples using flow cytometry" 489-522 *in* "Methods in Cell Biology-Flow Cytometry", 2nd Ed., vol 42 (1994) Academic Press Inc (New York)<br>entire chapter | 1-16 |
| A | Laxer M.A. et al. "DNA Sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction" Am. J. Trop. Med. Hyg., 45(6), 1991, 688-694<br>entire document | 1-16 |
| A | Webster K.A. et al., "Detection of *Cryptosporidium parvum* using a specific polymerase chain reaction" Vet. Parasitol., 50 (1993) 35-44<br>entire document | 1-16 |
| A | WO 94/02635 A (THE MINISTER OF AGRICULTURE FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND) 3 February 1994<br>entire document | 1-16 |

**INTERNATIONAL SEARCH REPORT**
Information on patent family members

International Application No.
PCT/AU 96/00274

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| WO | 9504749 | AU | 75598/94 | | | | |
| WO | 9502605 | AU | 73645/94 | CA | 2167295 | EP | 708782 |
| WO | 9405333 | EP<br>NO | 662003<br>950800 | FI | 950948 | HU | 69922 |
| EP | 544212 | JP | 5149949 | | | | |
| WO | 9108312 | AU | 69669/91 | EP | 502994 | EP | 662518 |
| WO | 9106643 | EP | 497798 | US | 5071743 | | |
| EP | 206769 | JP | 62006689 | | | | |
| WO | 8605805 | AU<br>US | 56976/86<br>5017493 | DK | 1525/85 | EP | 218652 |
| WO | 9402635 | EP | 652974 | | | | |
| WO | 9212992 | AU | 14597/92 | EP | 571554 | | |
| WO | 9202625 | AU | 83311/91 | EP | 548095 | | |

END OF ANNEX